APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1983, p. 308–309 Vol. 45, No. 1
0099-2240/83/010308-02$02.00/0

Enhancement of the 7α-Dehydroxylase Activity of a Gram-Positive Intestinal Anaerobe by Flavins

NORIYUKI MASUDA,[1]* HIROSHI ODA,[1] SEIJU HIRANO,[1] AND HIROMITSU TANAKA[2]

Department of Bacteriology[1] and First Department of Internal Medicine,[2] Faculty of Medicine, Kagoshima University, Kagoshima 890, Japan

Received 26 July 1982/Accepted 21 September 1982

The addition of flavins to the growth medium specifically enhanced the 7α-dehydroxylation of bile acids by anaerobically growing cultures of a *Eubacterium lentum*-like intestinal anaerobe, strain c-25, without an increase in cell growth. The order of the enhancement of the reaction was flavin adenine dinucleotide > flavin mononucleotide ≫ riboflavin.

7α-Dehydroxylation of cholic acid and chenodeoxycholic acid, yielding deoxycholic acid and lithocholic acid, respectively, is very important in the bacterial transformation of bile acids (6). Generally, this property is believed to be rare among intestinal microorganisms. Indeed, 7α-dehydroxylase activity has been found only in certain strains of anaerobic lactobacilli (6), *Clostridium leptum* (9), *Clostridium bifermentans* (7), *Clostridium sordellii* (7), *Bacteroides* spp. (3), and *Eubacterium* sp. (8, 12). Nevertheless, more than 80% of the fecal bile acids are dehydroxylated, indicating the extensive occurrence of this reaction in the gut. Consequently, the 7α-dehydroxylase may be assumed to be of phenomenally high activity. However, only feeble activity is usually observed in in vitro cultures of the organisms. Little is known about the biochemical factors responsible for the prevalence of this reaction in vivo. In this study, we examined the effect of chemical factors on 7α-dehydroxylation by anaerobically growing cultures of a *Eubacterium lentum*-like intestinal anaerobe, strain c-25.

Strain c-25 is a *E. lentum*-like intestinal anaerobe previously isolated and characterized (7) and maintained in GAM semisolid agar (10) (Nissui Pharmaceutical Co.). For biotransformation experiments, the strain was subcultured in GAM broth, and 0.1 ml of an overnight culture was inoculated into 4.0 ml of a modified peptone-yeast extract broth (modified PY broth) (7) containing 150 μg of bile acid per ml. After incubation at 37°C for 4 days in an anaerobic jar under nitrogen, the spent culture medium was acidified to pH 2.0 with 6 N HCl for extraction of bile acids. Bile acids extracted with ethyl acetate were methylated by the methanol-sulfuric acid procedure and analyzed by gas-liquid chromatography by means of a 3% QF-1 column as previously described (7). Bile acids are referred to by abbreviations of their trivial names: CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholanoic acid); 7OD, 7-oxodeoxycholic acid (3α,12α-dihydroxy-7-oxo-5β-cholanoic acid); and 7OL, 7-oxolithocholic acid (3α-hydroxy-7-oxo-5β-cholanoic acid). The bile acids used as substrates were CDCA (99.9% pure), a gift from Tokyo Tanabe Pharmaceutical Co., and CA (99.1% pure), purchased from Sigma Chemical Co. Riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) were from Nakarai Chemicals, Ltd., and NAD and NADP were from Sigma Chemical Co. Aqueous solutions were sterilized by filtration and added aseptically to the autoclaved assay medium at the desired concentrations.

Strain c-25 7α-dehydroxylated both CA and CDCA, yielding DCA and LCA, respectively, and also catalyzed 7α-dehydrogenation, giving rise to the formation of 7-oxo bile acids. However, only traces of these metabolites could be detected in a plain modified PY broth. In view of the recent reports on the role of FMN as an electron acceptor for 21-dehydroxylase activity (4, 5), c-25 was grown in the presence of various concentrations of this agent (Table 1). Progressive enhancement of the 7α-dehydroxylation was caused by increasing the amount of FMN, and almost complete dehydroxylation of CA and CDCA was achieved with a concentration of FMN equivalent to that of the substrate bile acids. Several flavin and nicotinamide derivatives were then added to the growth medium, and their enhancing effects were compared (Table 2). FMN and FAD specifically enhanced 7α-dehydroxylation (but not 7α-dehydrogenation), riboflavin was weakly stimulating, and no enhancement was observed with NAD and NADP. No increase in bacterial growth resulted from

TABLE 1. Enhancing effect of FMN on the 7α-dehydroxylation of CDCA and CA by strain c-25

Additive[a] (μg/ml)	% of CDCA[b] converted to:		% of CA converted to:	
	LCA	7OL	DCA	7OD
None	12	33	33	26
FMN				
1.5	17	29	63	17
15	38	20	59	20
75	Not tested	Not tested	83	7
150	86	4	90	5

[a] FMN was added to 4 ml of a modified PY broth containing 150 μg of CDCA or CA per ml at the indicated concentrations.

[b] After 4 days of anaerobic incubation at 37°C with c-25, the spent culture media were assayed for bile acids; the 7α-dehydroxylation (LCA or DCA) and 7α-dehydrogenation (7OL or 7OD) products are given as percentage conversion. All figures are the averages from triplicate tests.

the addition of these agents. Even when FMN was added at a concentration of 150 μg/ml to uninoculated media containing CA and CDCA, no transformation of bile acids was observed after anaerobic incubation at 37°C for 4 days. All the other reagents, cysteine, thioglycolate, hemin, and menadione, were ineffective.

The addition of flavin derivatives to the growth medium specifically enhanced the 7α-dehydroxylation of bile acids by a *E. lentum*-like intestinal anaerobe, c-25. However, cell growth was not stimulated by the addition of flavins, thus denying a nutritional role. In reference to recent reports showing the requirement of reduced flavins for the 21-dehydroxylation of corticosteroid by a strain of *E. lentum* (V.P.I. 11122) (4, 5), the results obtained here suggest that reduced flavins are required as electron donors in the reductive process of 7α-dehydroxylation. The shortage of these cofactors is a cause of the low 7α-dehydroxylase activity of the organism in plain modified PY broth. Since microbial cells are relatively impermeable to external flavins (2), exogenously added flavins may not completely penetrate the cell membrane. However, the permeation of these cofactors is not necessarily needed for the enhancement of the activity, because the reaction is thought to occur in or on the cytoplasmic membrane (9). Finally, Aries and Hill (1) reported that the activity is increased if cysteine or whole or lysed blood is added to the growth medium. White et al. (11) stated that NAD is the only cofactor that stimulates the activity in cell extracts of *Eubacterium* sp. strain VPI 12708. In contrast to these findings, none of these substrates affected the dehydroxylation by anaerobically growing cultures of c-25.

TABLE 2. Effect of flavin and nicotinamide derivatives on the 7α-dehydroxylation of CDCA by strain c-25

Additive[a]	% of CDCA[b] converted to:	
	LCA	7OL
None	7	28
Riboflavin (93)	25	17
FMN (113)	62	13
FAD (196)	87	3
NAD (179)	8	32
NADP (207)	7	29

[a] Each derivative was added at 1 μmol per tube (4.0 ml of the assay medium), which is shown as micrograms per milliliter in parentheses.

[b] Tests were performed as described in footnote *b* of Table 1.

We thank K. Nakazono for excellent technical assistance and Mary L. Robbins for careful review of the manuscript.

LITERATURE CITED

1. **Aries, V., and M. J. Hill.** 1970. Degradation of steroids by intestinal bacteria. II. Enzymes catalysing the oxidoreduction of the 3α, 7α- and 12α-hydroxyl groups in cholic acid, and the dehydroxylation of the 7-hydroxyl group. Biochim. Biophys. Acta **202**:535–543.
2. **Demain, A. L.** 1972. Riboflavin oversynthesis. Annu. Rev. Microbiol. **26**:369–388.
3. **Edenharder, R., and J. Slemrova.** 1976. Die Bedeutung des bakteriellen Steroidabbaus für die Ätiologie des Dickdarmkrebses. IV. Spaltung von Glykocholsäure, Oxydation und Reduktion von Cholsäure durch saccharolytische *Bacteroides*-Arten. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B **162**:350–373.
4. Feighner, S. D., V. D. Bokkenheuser, J. Winter, and P. B. Hylemon. 1979. Characterization of a C_{21} neutral steroid hormone transforming enzyme, 21-dehydroxylase, in crude cell extracts of *Eubacterium lentum*. Biochim. Biophys. Acta **574**:154–163.
5. **Feighner, S. D., and P. B. Hylemon.** 1980. Characterization of a corticosteroid 21-dehydroxylase from the intestinal anaerobic bacterium, *Eubacterium lentum*. J. Lipid Res. **21**:585–593.
6. **Hayakawa, S.** 1973. Microbiological transformation of bile acids. Adv. Lipid Res. **11**:143–192.
7. **Hirano, S., R. Nakama, M. Tamaki, N. Masuda, and H. Oda.** 1981. Isolation and characterization of thirteen intestinal microorganisms capable of 7α-dehydroxylating bile acids. Appl. Environ. Microbiol. **41**:737–745.
8. **Lipsky, R. H., and P. B. Hylemon.** 1980. Characterization of a NADH:flavin oxidoreductase induced by cholic acid in a 7α-dehydroxylating intestinal *Eubacterium* species. Biochim. Biophys. Acta **612**:328–336.
9. **Stellwag, E. J., and P. B. Hylemon.** 1979. 7α-Dehydroxylation of cholic acid and chenodeoxycholic acid by *Clostridium leptum*. J. Lipid Res. **20**:325–333.
10. **Sutter, V. L., D. M. Citron, and S. M. Finegold.** 1980. Wadsworth anaerobic bacteriology manual, 3rd ed., p. 98. C. V. Mosby Co., St. Louis, Mo.
11. **White, B. A., A. F. Cacciapuoti, R. J. Fricke, T. R. Whitehead, E. H. Mosbach, and P. B. Hylemon.** 1981. Cofactor requirements for 7α-dehydroxylation of cholic and chenodeoxycholic acid in cell extracts of the intestinal anaerobic bacterium, *Eubacterium* species V.P.I. 12708. J. Lipid Res. **22**:891–898.
12. **White, B. A., R. L. Lipsky, R. J. Fricke, and P. B. Hylemon.** 1980. Bile acid induction specificity of 7α-dehydroxylase activity in an intestinal *Eubacterium* species. Steroids **35**:103–109.